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Methanosarcina acetivorans sp. nov., an Acetotrophic Methane-Producing Bacterium Isolated from Marine Sediments

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A new acetotrophic marine methane-producing bacterium that was isolated from the methane-evolving sediments of a marine canyon is described. Exponential phase cultures grown with sodium acetate contained irregularly shaped cocci that aggregated in the early stationary phase and finally differentiated into communal cysts that released individual cocci when ruptured or transferred to fresh medium. The irregularly shaped cocci (1.9 ± 0.2 mm in diameter) were gram negative and occurred singly or in pairs. Cells were nonmotile, but possessed a single fimbria-like structure. Micrographs of thin sections showed a monolayered cell wall approximately 10 nm thick that consisted of protein subunits. The cells in aggregates were separated by visible septation. The communal cysts contained several single cocci encased in a common envelope. An amorphous form of the communal cyst that had incomplete septation and internal membrane-like vesicles was also present in late exponential phase cultures. Sodium acetate, methanol, methylamine, dimethylamine, and trimethylamine were substrates for growth and methanogenesis; H_2–CO_2 (80:20) and sodium formate were not. The optimal growth temperature was 35 to 40°C. The optimal pH range was 6.5 to 7.0. Both NaCl and Mg^{2+} were required for growth, with maximum growth rates at 0.2 M NaCl and 0.05 M $MgSO_4$. The DNA base composition was 41 ± 1% guanine plus cytosine. *Methanosarcina acetivorans* is the proposed species. C2A is the type strain (DSM 2834, ATCC 35395).

Low rates of methanogenesis occur in sulfate-rich marine sediments where sulfate reduction is the predominant terminal process in the anaerobic degradation of organic matter (34, 35). Methanogenesis predominates in marine environments where sulfate is readily depleted, which include the lower depths of marine sediments (24), sediments that receive large amounts of organic matter (26), and the elevated portions of marine marshes (14). Although acetate is the major precursor for methanogenesis in these sulfate-depleted sediments (31), marine acetotrophic methane-producing bacteria have not been isolated.

Several methanogenic bacteria that utilize H_2 or formate for growth have been isolated from marine sediments (7, 9, 11, 28–30, 39, 40). Recently, two marine methylotrophic species, "*Methanococcoides methylutens*" (36) and *Methanolobus tindarius* (15), were described, but neither utilizes acetate for growth. We describe here the first marine methanogenic bacterium reported that utilizes acetate in addition to methanol and methylated amines as a substrate for growth and methanogenesis.

MATERIALS AND METHODS

Source of inoculum. Marine sediment was obtained as described previously (36) from the Sumner branch of Scripps Canyon located near La Jolla, Calif.

Media. Sterile media were prepared under an N_2–CO_2 (80:20) atmosphere by a modification of the Hungate technique (2). All gases were passed through a column of reduced copper filings at 350°C to remove traces of O_2. Enrichment medium was as described previously (36) with 0.02 M sodium acetate as the substrate. The enrichment medium contained artificial seawater supplemented with the following constituents at the indicated final percent compositions (weight/volume): NH_4Cl, 0.05; Na_2CO_3, 0.1; Na_2HPO_4, 0.035; NaH_2PO_4, 0.030; cysteine-HCl · H_2O, 0.025; Na_2S · $9H_2O$, 0.025; $FeSO_4$, 0.001; resazurin, 0.0001. In addition, 1% (vol/vol) vitamin solution and 1% (vol/vol) trace element solution were added (43). The final pH of the medium was 7.2. Roll tubes contained enrichment medium with the addition of 2% purified agar. Maintenance medium (36) contained the following constituents at the indicated final percent compositions (weight/volume) in demineralized water: NaCl, 2.34; $MgSO_4$, 0.63; Na_2CO_3, 0.5; yeast extract, 0.1; NH_4Cl, 0.05; KCl, 0.08; $CaCl_2$ · $2H_2O$, 0.014; Na_2HPO_4, 0.06; cysteine-HCl · H_2O, 0.025; Na_2S · $9H_2O$, 0.025; resazurin, 0.0001. In addition, 1% (vol/vol) each of vitamin and trace element solutions (43) and 0.05 M of the indicated substrate were added to the medium. The final pH of the medium was 7.2. Strains were maintained on agar slants of maintenance medium with sodium acetate contained in 16- by 150-mm anaerobe tubes (Bellco Glass, Inc., Vineland, N.J.) that were sealed with butyl rubber stoppers.

Enrichments and isolation. Enrichments were started by adding sediment (5 ml) to 160-ml serum vials (Wheaton Scientific, Millville, N.J.) that contained 45 ml of enrichment medium. The vials were purged with N_2–CO_2 (80:20) and sealed with butyl rubber septa secured by aluminum crimp collars (2). The cultures were incubated in the dark at 25°C. After methane production subsided, 5 ml of the culture was anaerobically transferred into a new vial of sterile enrichment medium. This procedure was repeated for five successive transfers, and then 1 ml of the culture was transferred to an agar slant of maintenance medium that contained 0.05 M sodium acetate and vancomycin (100 mg/liter). The use of agar slants enhanced growth, and the vancomycin inhibited the growth of nonmethanogenic organisms. When the liquid of syneresis became turbid, serial dilutions of the culture were inoculated into tubes (25 by 150 mm) of molten agar maintenance medium containing sodium acetate and then rolled (4). Colonies were picked with a bent, sterile Pasteur pipette. The plug of agar containing a colony was transferred to the liquid of syneresis in a sterile agar slant of maintenance medium. All strains were maintained by monthly transfer on agar slants incubated at 20°C.

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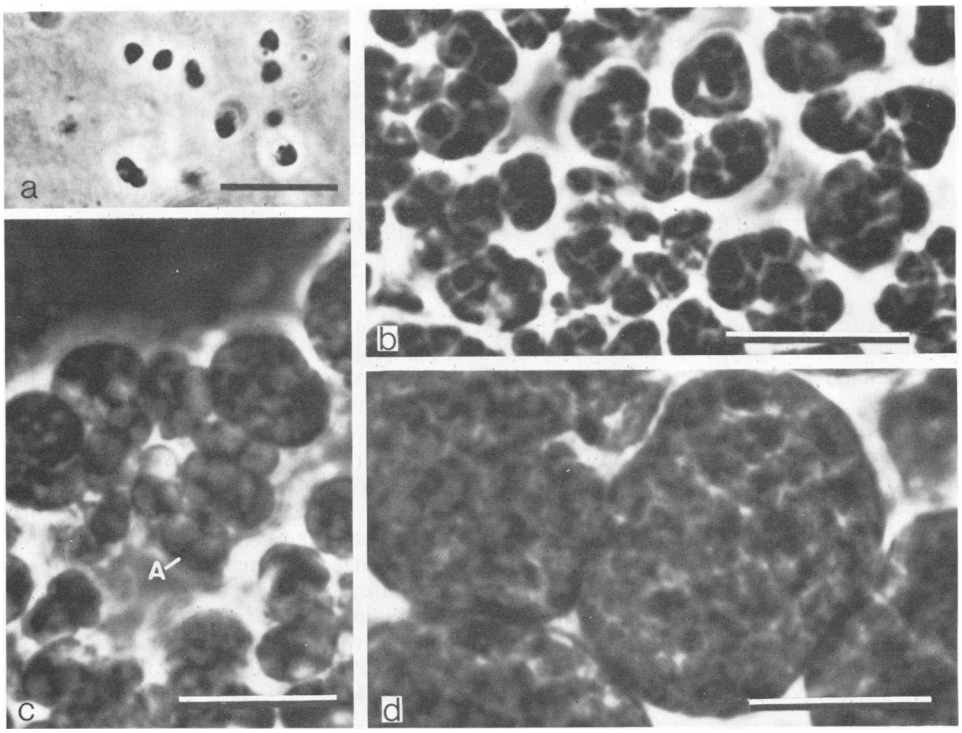

FIG. 1. Phase-contrast micrographs of strain C2A grown on acetate. (a) Single cells from an exponential phase culture (bar, 5 μm). (b) Cell aggregates from a late exponential phase culture (bar, 10 μm). (c) Aggregates of large cells (A) (bar, 10 μm). (d) Communal cysts from a late exponential phase culture (bar, 10 μm).

Large-scale production of cell material. Cells for biochemical characterization and determination of the DNA base composition were grown in a 12-liter fermentor (model MP-114; New Brunswick Scientific Co., Inc., Edison, N.J.) that contained 10 liters of maintenance medium. The fermentor was operated as a pH-stat (32) with acetic acid as the growth substrate.

Growth methods. The most probable number of acetotrophic methane-producing bacteria in sediments was determined as described previously (10) by using enrichment medium.

Growth experiments were performed in culture tubes (16 by 150 mm; Bellco Glass, Inc.) that contained 10 ml of maintenance medium. Trimethylamine (0.05 M) was used as the substrate to avoid the formation of aggregates. The tubes were sealed with butyl rubber septa secured with aluminum crimp collars. Cultures were anaerobically transferred with a syringe (2). Cultures were incubated at 40°C. Growth was followed at 550 nm with a Spectronic 20 spectrophotometer (Bausch & Lomb Inc., Rochester, N.Y.). The results are reported as the mean of values from triplicate cultures.

Glassware for growth factor experiments was cleaned in concentrated sulfuric acid, rinsed three times with tap water and six times with deionized water, and then ashed. Media were prepared with demineralized water that was passed at 5 ml/min through a 1.5- by 18-cm column that contained activated charcoal (Bio-Rad Laboratories, Richmond, Calif.). The culture tubes were sealed with new butyl rubber septa that had been rinsed with charcoal-treated water.

Growth at each indicated pH was determined by substituting for sodium carbonate the following buffers at a final concentration of 0.1 M: pH 5.5 and 6.0, 2(*N*-morpholino)ethanesulfonic acid (pK_a, 6.1); pH 6.5 and 7.0, piperazine *N*-*N*′-bis(2-ethanesulfonic acid) monosodium monohydrate (pK_a, 6.8); pH 7.5, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pK_a, 7.5); pH 8.0 and 8.5, *N*,*N*-bis(2 hydroxyethyl)glycine (pK_a, 8.3). All cultures were incubated at 35°C.

Molar growth yields were determined during exponential growth in a pH-stat. The dry weight was determined by fixing 10 ml of culture material with 2% glutaraldehyde for 15 min and then filtering the cells on a predried filter (0.2-μm pore size; Millipore Corp., Bedford, Mass.). The cells and filter were washed with deionized water, dried overnight at 90°C, and weighed. One optical density unit corresponded to a cell mass of 0.323 g (dry weight) per liter.

Analytical methods. Coenzyme F_{420} was partially purified from cell extract by DEAE-cellulose column chromatography, and its concentration was determined spectrophotometrically (32). Protein was determined by the Bradford assay with bovine serum albumin as a standard (3). Cell walls were isolated, and the amino acids and amino sugars were analyzed as described previously (36). Methane was assayed with a gas chromatograph equipped with a flame ionization detector (36).

Moles percent guanine plus cytosine. Cells were lysed with sodium dodecyl sulfate (SDS). DNA was isolated and purified by the procedure of Marmur (23). The moles percent G+C was determined by thermal denaturation with a spectrophotometer and thermal programmer (model 2400; Gilford Instrument Laboratories, Inc., Oberlin, Ohio) (8). DNAs from *Bacteroides fragilis* (VPI 2553) and *Escherichia coli* b were used as standards.

Microscopy. Phase-contrast micrographs were made with a Leitz Dialux microscope. Negative stains and thin-section electron micrographs were prepared as described previously

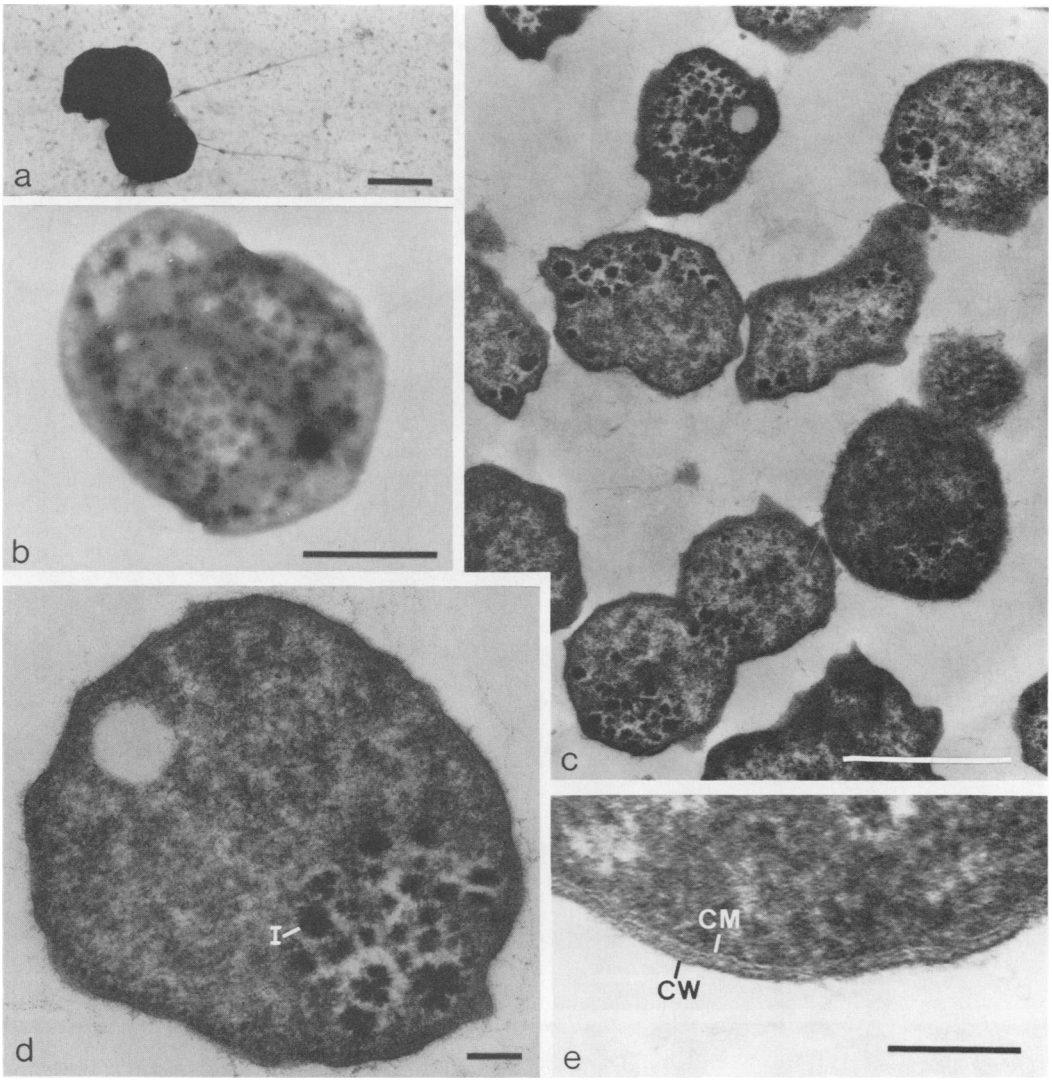

FIG. 2. Electron micrographs of strain C2A single cells grown on acetate. (a) Negative stain of whole cells with fimbria-like structures (bar, 1 μm). (b) Negative stain of a whole cell showing irregular shape and inclusions (bar, 0.5 μm). (c and d) Thin sections showing inclusions (I) (c: bar, 1 μm; d: bar, 0.2 μm). (e) Thin section showing the cell wall (CW) and cytoplasmic membrane (CM) (bar, 100 nm).

(36). The procedure for UV fluorescence microscopy was as described previously (25) with a Leitz Orthoplan fluorescence microscope.

Chemicals. Purified agar and yeast extract were from Difco Laboratories (Detroit, Mich.). Trypticase was from BBL Microbiology Systems (Cockeysville, Md.). Activated charcoal was from Bio-Rad. Trimethylamine, dimethylamine, and methylamine hydrochloride were obtained from Aldrich Chemical Co. (Milwaukee, Wis.). Bovine serum albumin, vancomycin, RNases A and T_1, DNase I, Triton X-100, SDS, and all organic buffers and vitamins were obtained from Sigma Chemical Co. (St. Louis, Mo.). Vitamin-free, salt-free Casamino Acids were from ICN Nutritional Biochemicals (Cleveland, Ohio). All other chemicals were reagent grade.

RESULTS

Enrichment and isolation. A survey of sediment samples by a most probable number dilution series indicated that the acetotrophic methane-producing population ranged from 530 to 4,300/cm^3 in the first 20 cm of a depth profile. Strain C2A was isolated from an enrichment culture inoculated with the upper 10 cm of this sediment. The predominant organisms in this enrichment were irregularly shaped cocci that occurred singly or in pairs and fluoresced blue-green when examined by UV fluorescence microscopy. Colonies in roll tubes were pale yellow and were 0.5 mm in diameter after 14 days of incubation. Surface colonies were smooth, circular, and convex with entire edges. Several colonies of irregular cocci were isolated, and strain C2A was selected for further study.

Cell morphology. Acetate-grown cultures of strain C2A in the exponential phase contained irregular cocci with an average diameter of 1.9 ± 0.2 μm (Fig. 1a). A single fimbria-like appendage was visible in electron micrographs of negatively stained cells (Fig. 2a). These structures did not have the helical appearance of flagella, and motility was not observed in wet mounts. Electron-dense granules were seen in electron micrographs of negatively stained whole cells

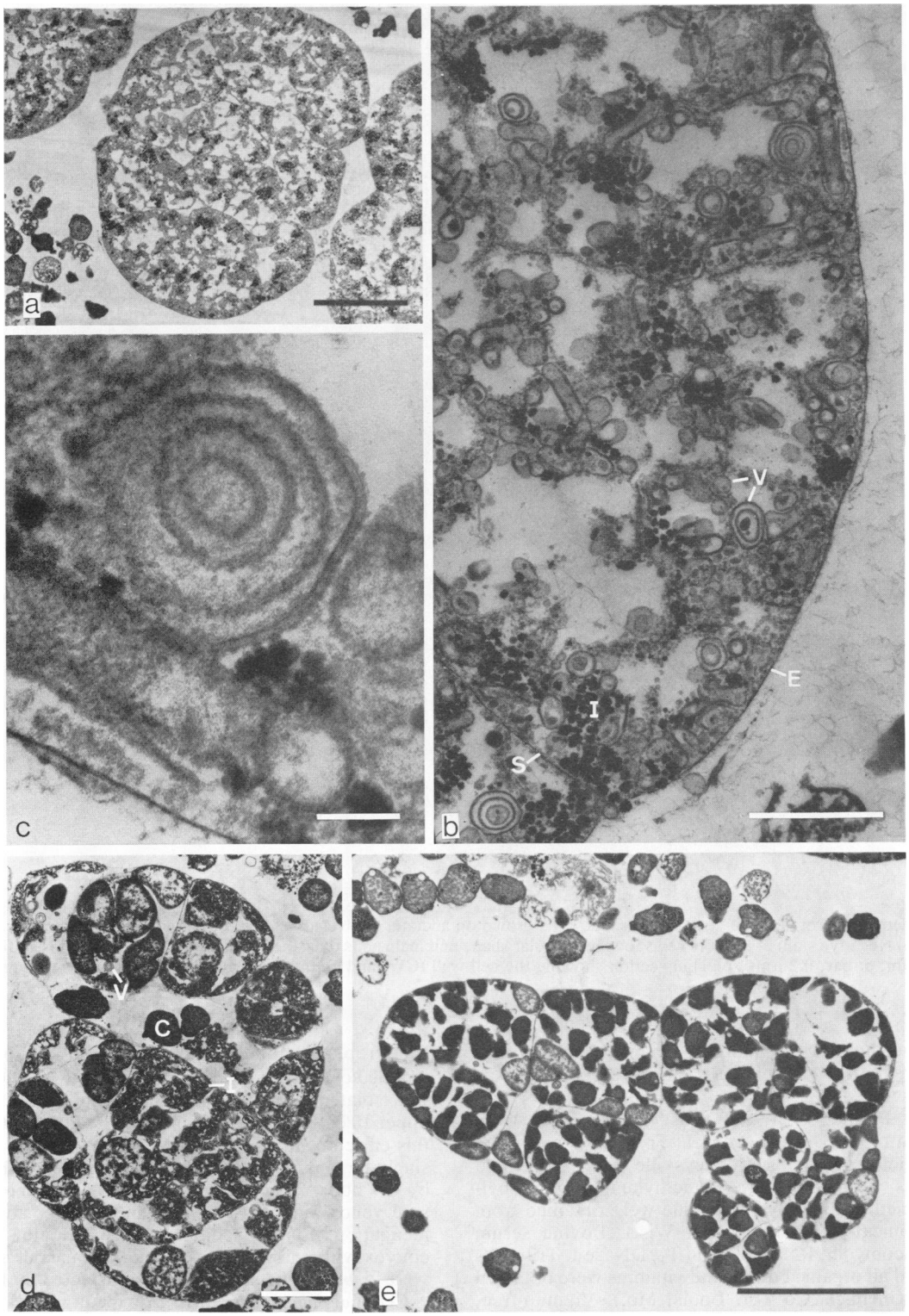

FIG. 3. Electron micrographs of strain C2A cysts. (a and b) Thin sections of the amorphous form showing cytoplasmic inclusions (I), internal vesicles (V), incomplete septation (S), and the external envelope (E) (a: bar, 5 μm; b: bar, 1 μm). (c) Thin section of the amorphous form showing internal vesicles with apparent subunit structure (bar, 100 nm). (d) Thin section showing an incompletely formed communal cyst containing individual cells (C), cytoplasmic inclusions (I), and internal vesicles (V) (bar, 2 μm). (e) Thin section of a communal cyst containing only whole cells enclosed by a common envelope (bar, 5 μm).

(Fig. 2b) and thin sections (Fig. 2c and d). Although cells stained gram negative, thin sections revealed a monolayer cell wall 10 nm thick that is characteristic of described marine methanogenic bacteria with a protein cell wall (7, 9, 11, 15, 28–30, 36, 39, 40) (Fig. 2e). Pelleted cells were lysed immediately when suspended in maintenance medium that contained SDS (0.005%, wt/vol) or Triton X-100 (0.01%, vol/vol). Lysis also occurred when cells were suspended in maintenance medium from which either NaCl or $MgSO_4$ was omitted or when $MnSO_4$ was substituted for $MgSO_4$. Lysis did not occur when KCl was substituted for NaCl. Acid-hydrolyzed cell wall preparations contained a wide distribution of amino acids, which accounted for 98% of the organic fraction (Table 1). No amino sugars were detected. These results show that strain C2A contains an osmotically fragile protein cell wall.

As acetate-grown cultures approached the stationary phase, aggregates of 2 to 12 cells predominated (Fig. 1b). Loosely associated aggregates of larger cells (3 to 4 μm in diameter) were also present (Fig. 1c). The predominant form in stationary phase cultures was a communal cyst composed of many single cocci within a common envelope (Fig. 1d). When these communal cysts were ruptured by applying pressure to the cover slip, single, irregular cocci were released that resembled cells from exponential phase cultures. These cysts ruptured and released single cocci several days after transfer to fresh medium that contained acetate. The release of individual cocci occurred sooner when the cysts were transferred to medium that contained methanol or methylated amines. Aggregates or communal cysts were not present in cultures grown with methanol or methylated amines.

Thin-section electron micrographs of late exponential phase, acetate-grown cultures showed an amorphous form of the communal cyst. This form contained incomplete septation, electron-dense granules, and internal structures that resembled membrane vesicles (Fig. 3a and b). All of these components were enclosed within a common envelope approximately 6 nm thick. The internal membranes were approximately 10 nm thick and appeared to have subunit structure (Fig. 3c). These membranes appeared as concentric rings in cross section, which indicated that they may be closed vesicles. Some cysts contained both single cells and components of the amorphous form within a common envelope (Fig. 3d). Communal cysts contained fully developed cells all surrounded by a thin (6-nm) envelope (Fig. 3e).

TABLE 1. Amino acid content of the hydrolyzed cell wall preparation of strain C2A

Amino acid	μmol/mg (dry wt)	Molar ratio (His = 1.0)
Glu	0.326	5.66
Ala	0.319	5.53
Gly	0.307	5.32
Asp	0.292	5.07
Leu	0.242	4.20
Lys	0.196	3.40
Ile	0.172	2.98
Val	0.157	2.72
Pro	0.145	2.52
Thr	0.145	2.51
Ser	0.136	2.37
Arg	0.131	2.27
Met	0.102	1.76
Phe	0.096	1.67
Tyr	0.074	1.28
His	0.062	1.00
Cys	0.029	0.51
Trp	ND[a]	ND

[a] ND, Not determined.

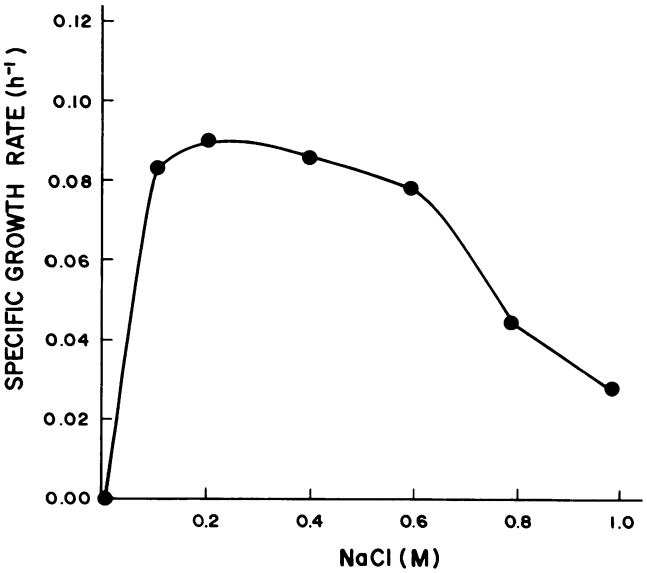

FIG. 4. Effect of NaCl concentration on the growth rate of strain C2A with trimethylamine. K_2CO_3 and K_2HPO_4 were substituted for Na_2CO_3 and Na_2HPO_4. The final Na^+ concentration included Na_2S in the medium.

Physiology. Sodium acetate, methanol, methylamine, dimethylamine, and trimethylamine supported growth, with doubling times of 24.1, 5.2, 6.7, 7.8, and 7.3 h, respectively. Acetate-grown cells did not grow or produce methane when transferred to medium that contained H_2–CO_2 (80:20) or sodium formate as the sole substrate. The molar growth yield of strain C2A was 2.4 g (dry weight) per mol of acetate consumed.

Strain C2A did not require exogenous growth factors. Trypticase or vitamins (42) did not stimulate growth, but yeast extract or vitamin-free Casamino Acids were slightly stimulatory (data not shown). The optimal NaCl range for growth was between 0.1 and 0.6 M (Fig. 4). No growth occurred without NaCl. Neither Na_2SO_4 nor KCl could be substituted for NaCl, which indicated that both Na^+ and Cl^- were required. Maximum growth rates occurred with $MgSO_4$ concentrations between 0.05 and 0.1 M (Fig. 5). No growth occurred in the absence of $MgSO_4$. $MgCl_2$ substituted for $MgSO_4$, which indicated that only Mg^{2+} was required. Growth occurred over a pH range of 5.5 to 8.0, and the maximum rate of growth was near pH 6.5 (Fig. 6). No significant inhibition was observed with any of the buffers tested, and the pH of the cultures did not change significantly during growth. The maximum rate of growth was at 40°C, and no growth was detected below 10°C or above 50°C (Fig. 7).

Cell extracts of acetate-grown cells contained 46 ng of coenzyme F_{420} per mg of protein. The DNA base composition was 41 ± 1 mol% G+C.

DISCUSSION

Methanogenic isolates that utilize H_2 or formate have been described from a diversity of marine habitats (7, 9, 11, 28–30, 39, 40). Recently, two methylotrophic methane-producing marine isolates were described that utilize methylated

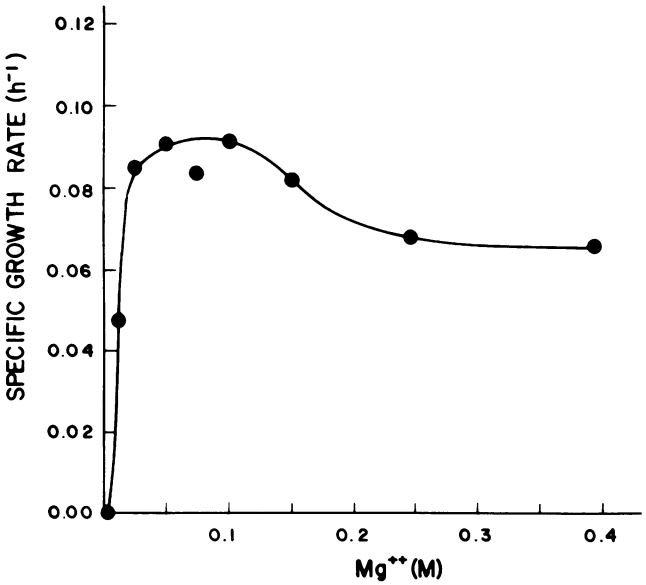

FIG. 5. Effect of Mg^{2+} on the growth rate of strain C2A with trimethylamine.

amines and methanol, but not acetate (15, 36). Strain C2A is the first marine methanogenic bacterium described that utilizes acetate as a substrate.

Methanogenesis has been reported in a variety of marine sediments. Low rates of methane production occur in sulfate-rich marine sediments (34, 35), where sulfate-reducing bacteria outcompete methanogenic organisms for H_2 and acetate (16, 17, 19, 21, 33). The methane produced in these sulfate-rich marine sediments may also be derived from methylated amines, which are not utilized by the sulfate-reducing bacteria (13, 27, 41). Methane production predominates in sulfate-depleted marine sediments that receive high organic loading (26) and in the tidal zones of marshes (14). Acetate is a major substrate for methanogenesis in nonmarine sediments (5, 20) and may also be a major substrate for methane production in sulfate-depleted marine sediments (31). Strain C2A was isolated from a marine canyon with large deposits of kelp and sea grass where acetotrophic methane-producing bacteria like strain C2A may be important in the degradation of organic matter.

The morphology of strain C2A changed during growth on acetate and depended upon the phase of growth. Only single cocci were present in exponential phase cultures. As cultures approached late exponential phase, methanosarcina-like cell aggregates appeared among the single cocci. These aggregates developed into communal cysts composed of a loose arrangement of single cocci enclosed within a common envelope. These cysts were often present in old cultures and may provide a means of survival. Late exponential phase cultures also contained a morphological form that resembled a communal cyst, but had incomplete septation and internal membrane-like structures. This amorphous form may be an intermediate stage between the aggregate and communal cyst.

Although the various cell morphologies of strain C2A were similar to the acetotrophic isolates *Methanosarcina mazei* (22) and *Methanosarcina* sp. of morphovar 3 (43), there were significant differences. The cell wall of strain C2A was thinner than that of *Methanosarcina* sp. of morphovar 3 and consisted of protein rather than the SDS-insensitive heteropolysaccharide present in *M. mazei* cell walls (22; S. F. Hurst, R. W. Robinson, and A. S. Bleiweis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, J10, p. 92). "Microcysts" and "pseudococci," reported in cultures of *Methanosarcina* sp. of morphovar 3, were not present in cultures of strain C2A. Communal cysts of strain C2A were unlike the macrocyst described for *Methanosarcina* sp. of morphovar 3, which contained tightly packed pseudococci and "polygonal" cells (43).

Like all other described marine methanogenic bacteria (1, 7, 9, 11, 15, 28–30, 36, 39, 40), strain C2A is gram negative and SDS sensitive and has a protein cell wall. Protein cell walls are found only in certain members of the archaeobacteria, which include methanogenic bacteria, extreme halophiles, and thermophilic sulfur-oxidizing bacteria (12, 37, 38). The cell wall of strain C2A contained a high proportion of the negatively charged amino acids aspartate and glutamate, a feature found in all described archaeobacterial protein cell walls (12, 36–38). The ability to lyse strain C2A by the substitution of $MnSO_4$ for $MgSO_4$ indicates that Mg^{2+} is required to maintain the integrity of the cell wall. Perhaps Mg^{2+} maintains the integrity of the cell wall by binding loosely with cell wall proteins to reduce the electrostatic repulsions between the negatively charged subunits (18, 37). The inability to substitute another divalent cation for Mg^{2+} may result from differences in their binding properties. Lysis in the absence of NaCl appears to be an osmotic effect since KCl could be substituted for NaCl. The ability to gently lyse strain C2A by elimination of NaCl or $MgSO_4$ or by treatment with SDS is advantageous for biochemical and genetic studies of methylotrophic and acetotrophic metabolism.

Immunological fingerprinting (6) by indirect immunofluorescence (S-probe) showed that strain C2A cross-reacted with members of the *Methanosarcinaceae*, but did not cross-react completely with presently described strains (E. Conway de Macario, personal communication). rRNA hybridization studies showed that strain C2A had greater than 85% homology with described species of *Methanosarcina* (K. R. Sowers, J. L. Johnson, and J. G. Ferry, submitted for publication).

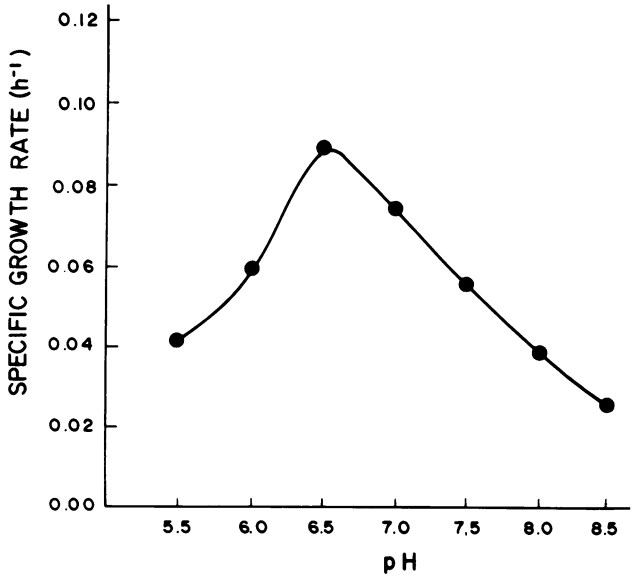

FIG. 6. Effect of pH on the growth rate of strain C2A with trimethylamine. See the text for the buffer used at each pH.

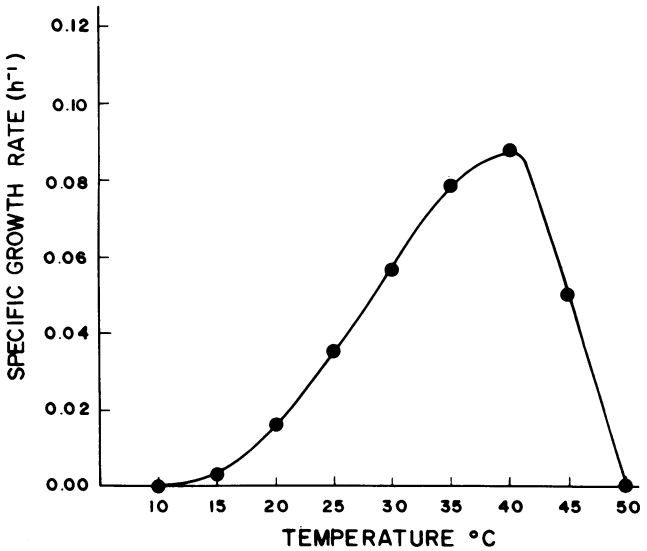

FIG. 7. Effect of temperature on the growth rate of strain C2A with trimethylamine.

We propose that strain C2A be placed in the family *Methanosarcinaceae* as described by Balch et al. (1), based on DNA-RNA homologies, immunological fingerprinting, and the ability to use acetate and other methylotrophic substrates. Phenotypic characteristics that distinguish this strain from members of the *Methanosarcina* are the presence of a gram-negative protein cell wall, SDS sensitivity, and a requirement for NaCl and Mg^{2+}. Characteristics that distinguish it from "*M. methylutens*" and *M. tindarius* are the ability to use acetate as a sole substrate and the formation of methanosarcina-like aggregates and communal cysts. We therefore propose that this strain be placed in a newly described species of methanogenic bacteria, *Methanosarcina acetivorans*. C2A is the type strain.

The following species description is proposed: *M. acetivorans* sp. nov. (a.ce.tí'vo.rans). L.n. *acetum*, vinegar; L. part. adj. *vorans*, consuming; N.L. mas. adj. *acetivorans*, consuming acetic acid.

Morphovars of this species, when grown on acetate, occur as irregular cocci, septated cell aggregates, or communal cysts that contain several individual cocci within a common envelope. Individual cocci are 1.9 μm in diameter and nonmotile. Surface colonies are pale yellow, circular, and convex with entire edges. Cells are lysed by SDS and possess a thin (10-nm), gram-negative cell wall. Acetate, methanol, methylamine, dimethylamine, and trimethylamine are growth substrates; H_2–CO_2 (80:20) and sodium formate are not; NaCl (0.2 M optimum) and Mg^{2+} (0.05 to 1.0 M optimum) are required for growth; no exogenous growth factors are required; the optimum temperature is 35 to 40°C; the optimum pH is 6.5 to 7.0; it is a strict anaerobe. The source is anaerobic sediments from the Sumner branch of Scripps Canyon located near La Jolla, California. The type strain is C2A. This strain has been deposited in the German Collection of Microorganisms, Göttingen, Germany, as DSM 2834 and in the American Type Culture Collection, Rockville, Md., as ATCC 35395.

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LITERATURE CITED

1. **Balch, W. E., G. E. Fox, L. J. Magrum, C. R. Woese, and R. S. Wolfe.** 1979. Methanogens: reevaluation of a unique biological group. Microbiol. Rev. **43**:260–296.
2. **Balch, W. E., and R. S. Wolfe.** 1976. New approach to the cultivation of methanogenic bacteria: 2-mercaptoethanesulfonic acid (HS-CoM)-dependent growth of *Methanobacterium ruminantium* in a pressurized atmosphere. Appl. Environ. Microbiol. **32**:781–791.
3. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
4. **Bryant, M. P.** 1972. Commentary on the Hungate technique for culture of anaerobic bacteria. Am. J. Clin. Nutr. **25**:1324–1328.
5. **Cappenberg, T. E., and R. A. Prins.** 1974. Interrelations between sulfate-reducing and methane-producing bacteria in bottom deposits of a fresh water lake. III. Experiments with ^{14}C-labeled substrates. Antonie van Leeuwenhoek J. Microbiol. Serol. **40**:457–469.
6. **Conway de Macario, E., A. J. L. Macario, and M. J. Wolin.** 1982. Specific antisera and immunological procedures for characterization of methanogenic bacteria. J. Bacteriol. **149**:320–328.
7. **Huber, H., M. Thomm, H. König, G. Thies, and K. Stetter.** 1982. *Methanococcus thermolithotrohicus*, a novel thermophilic lithotrophic methanogen. Arch. Microbiol. **132**:47–50.
8. **Johnson, J. L.** 1981. Genetic characterization, p. 450–472. *In* P. Gerhardt (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
9. **Jones, J. B., B. Bowers, and T. C. Stadtman.** 1977. *Methanococcus vannielii*: ultrastructure and sensitivity to detergents and antibiotics. J. Bacteriol. **130**:1357–1363.
10. **Jones, W. J., and M. J. B. Paynter.** 1980. Populations of methane-producing bacteria and in vitro methanogenesis in salt marsh and estuarine sediments. Appl. Environ. Microbiol. **39**:864–871.
11. **Jones, W. J., M. J. B. Paynter, and R. Gupta.** 1983. Characterization of *Methanococcus maripaludis* sp. nov., a new methanogen isolated from salt marsh sediment. Arch. Microbiol. **135**:91–97.
12. **Kandler, O., and H. König.** 1978. Chemical composition of the peptidoglycan-free cell walls of methanogenic bacteria. Arch. Microbiol. **118**:141–152.
13. **King, G. M., M. J. Klug, and D. R. Lovley.** 1983. Metabolism of acetate, methanol, and methylated amines in intertidal sediments of Lowes Cove, Maine. Appl. Environ. Microbiol. **45**:1848–1853.
14. **King, G. M., and W. J. Wiebe.** 1980. Tracer analysis of methanogenesis in salt marsh soils. Appl. Environ. Microbiol. **39**:877–881.
15. **König, H., and K. O. Stetter.** 1982. Isolation and characterization of *Methanolobus tindarius* sp. nov., a coccoid methanogen growing only on methanol and methylamines. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. **3**:478–490.
16. **Kristjansson, J. K., P. Schönenheit, and R. K. Thauer.** 1982. Different K_s values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: an explanation for the apparent inhibition of methanogenesis by sulfate. Arch. Microbiol. **131**:278–282.
17. **Laanbroek, H. J., and N. Pfennig.** 1981. Oxidation of short-chain fatty acids by sulfate-reducing bacteria in freshwater and marine sediments. Arch. Microbiol. **128**:330–335.
18. **Larsen, H.** 1963. Halophilism, p. 297–342. *In* I. C. Gunalus and R. Y. Steiner (ed.), The bacteria, vol 4. Academic Press, Inc., New York.
19. **Lovley, D. R., D. F. Dwyer, and M. J. Klug.** 1982. Kinetic analysis of competition between sulfate reducers and methano-

gens for hydrogen in sediments. Appl. Environ. Microbiol. **43**:1373–1379.
20. **Lovley, D. R., and M. J. Klug.** 1982. Intermediary metabolism of organic matter in the sediments of a eutrophic lake. Appl. Environ. Microbiol. **43**:552–560.
21. **Lovley, D. R., and M. J. Klug.** 1983. Sulfate reducers can outcompete methanogens at freshwater sulfate concentrations. Appl. Environ. Microbiol. **45**:187–192.
22. **Mah, R. A.** 1980. Isolation and characterization of *Methanococcus mazei*. Curr. Microbiol. **3**:321–326.
23. **Marmur, J.** 1961. A procedure for the isolation of deoxyribonucleic acid from microorganisms. J. Mol. Biol. **3**:208–218.
24. **Martens, C. S., and R. A. Berner.** 1974. Methane production in the interstitial waters of sulfate-depleted sediments. Science **185**:1167–1169.
25. **Mink, R. W., and P. R. Dugan.** 1977. Tentative identification of methanogenic bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:713–717.
26. **Mountfort, D. O., and R. A. Asher.** 1981. Role of sulfate reduction versus methanogenesis in terminal carbon flow in polluted intertidal sediment of Waimea Inlet, Nelson, New Zealand. Appl. Environ. Microbiol. **42**:252–258.
27. **Oremland, R. S., and S. Polcin.** 1982. Methanogenesis and sulfate reduction: competitive and noncompetitive substrates in estuarine sediments. Appl. Environ. Microbiol. **44**:1270–1276.
28. **Rivard, C. J., J. M. Henson, M. V. Thomas, and P. H. Smith.** 1983. Isolation and characterization of *Methanomicrobium paynteri* sp. nov., a mesophilic methanogen isolated from marine sediments. Appl. Environ. Microbiol. **46**:484–490.
29. **Rivard, C. J., and P. H. Smith.** 1982. Isolation and characterization of a thermophilic marine methanogenic bacterium, *Methanogenium thermophilicum* sp. nov. Int. J. Syst. Bacteriol. **32**:430–436.
30. **Romesser, J. A., R. S. Wolfe, F. Mayer, E. Spiess, and A. Walther-Mauruschat.** 1979. *Methanogenium*, a new genus of marine methanogenic bacteria, and characterization of *Methanogenium cariaci* sp. nov. and *Methanogenium marisnigri* sp. nov. Arch. Microbiol. **121**:147–153.
31. **Sansone, F. J., and C. S. Martens.** 1981. Methane production from acetate and associated methane fluxes from anoxic coastal sediments. Science **211**:707–709.
32. **Schauer, N. L., and J. G. Ferry.** 1980. Metabolism of formate in *Methanobacterium formicicum*. J. Bacteriol. **142**:800–807.
33. **Schönheit, P.** 1982. Kinetic mechanism for the ability of sulfate-reducers to out-compete methanogens for acetate. Arch. Microbiol. **132**:285–288.
34. **Senior, E., E. B. Lindström, I. M. Banat, and D. B. Nedwell.** 1982. Sulfate reduction and methanogenesis in the sediment of a saltmarsh on the East Coast of the United Kingdom. Appl. Environ. Microbiol. **43**:987–996.
35. **Sørensen, J., D. Christensen, and B. B. Jørgensen.** 1981. Volatile fatty acids and hydrogen as substrates for sulfate-reducing bacteria in anaerobic marine sediment. Appl. Environ. Microbiol. **42**:5–11.
36. **Sowers, K. R., and J. G. Ferry.** 1983. Isolation and characterization of a methylotrophic marine methanogen, *Methanococcoides methylutens* gen. nov., sp. nov. Appl. Environ. Microbiol. **45**:684–690.
37. **Steensland, H., and H. Larsen.** 1969. A study of the cell envelope of the *Halobacteria*. J. Gen. Microbiol. **55**:325–336.
38. **Weiss, R. L.** 1974. Subunit cell wall of *Sulfolobus acidocaldarius*. J. Bacteriol. **118**:275–284.
39. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149**:852–863.
40. **Wildgruber, G., M. Thomm, H. König, K. Ober, T. Ricchiuto, and K. O. Stetter.** 1982. *Methanoplanus limicola*, a plate-shaped methanogen representing a novel family, the *Methanoplanaceae*. Arch. Microbiol. **132**:31–36.
41. **Winfrey, M. R., and D. M. Ward.** 1983. Substrates for sulfate reduction and methane production in intertidal sediments. Appl. Environ. Microbiol. **45**:193–199.
42. **Wolin, E. A., M. J. Wolin, and R. S. Wolfe.** 1963. Formation of methane by bacterial extracts. J. Biol. Chem. **238**:2882–2886.
43. **Zhilina, T. N., and G. A. Zavarzin.** 1979. Cyst formation by *Methanosarcina*. Mikrobiologiya **48**:451–456.